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Regulation of Vav–SLP-76 Binding by ZAP-70 and Its Relevance to TCRζ/CD3 Induction of Interleukin-2

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Summary

T cell activation stimulates p56^{lck}, p59^{fyn}, ZAP-70, Vav–SLP-76 binding, and IL-2 transcription. Major questions concern the tyrosine-kinase and relevant site(s) needed for Vav–SLP-76 complex formation and its role in IL-2 production. Here, we show that of the three kinases, only ZAP-70 phosphorylates SLP-76 at specific sites that allow Vav SH2 domain binding. Therefore, while p56^{lck} regulates proximal events, ZAP-70 acts downstream on targets such as SLP-76. We also show by in vitro and in vivo analysis that two SLP-76 pYESP motifs (Y113 and Y128) mediate binding, the first being more efficient. A third pYEPP motif (Y145) failed to bind. Finally, TCRζ/CD3 ligation of T cell hybridoma DC27.10 induces IL-2 production without detectable Vav–SLP-76 binding. Therefore, despite effects of Vav–SLP-76 on IL-2 expression, Vav–SLP-76 binding per se is not essential for IL-2 production in all T cells.

Introduction

Ligation of the T cell receptor (TCRζ/CD3) and the CD4/CD8-p56^{lck} coreceptors activates Src protein-tyrosine kinases p56^{lck} and p59^{fyn} (Rudd et al., 1994), which in turn leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCRζ and CD3 chains (Samelson and Klausner, 1992; Weiss and Littman, 1994; Mustelin, 1994; Wange and Samelson, 1996). p56^{lck} phosphorylation of TCR components results in the recruitment of ZAP-70 via tandem Src homology 2 (SH2) domain binding (Wange et al., 1993; Iwashima et al., 1994) and possibly other proteins such as Shc (Ravichandran et al., 1993). ZAP-70 catalytic activity is then up-regulated by p56^{lck} phosphorylation (Chan et al., 1995; Wange et al., 1995). The activation of Src family and Syk/ZAP-70 protein-tyrosine kinases leads to phosphorylation of numerous cellular proteins, including CD5 (Burgess et al., 1992; Raab et al., 1994), phospholipase C-γ1 (PLC-γ1) (Weiss et al., 1991), the proto-oncogene Vav (Bustelo and Barbacid, 1992; Gulbins et al., 1993), p120/130 (da Silva et al., 1993, 1997), c-cbl (Donovan et al., 1994; Reedquist et al., 1996), and the recently cloned SLP-76 (Jackman et al., 1995). While tyrosine phosphorylation of PLC-γ1 regulates its enzymatic activity, leading to the activation of the inositol phospholipid pathway, the significance of the tyrosine phosphorylation of Vav and SLP-76 remains unclear.

The proto-oncogene Vav was first identified by its transforming activity in fibroblasts (Katzav et al., 1989). Vav is specifically expressed in hematopoietic cells and contains a number of structural motifs, including a putative guanine nucleotide exchange factor (Dbl) domain for the Rho/Rac/CDC42 family of small GTPases; a Pleckstrin homology domain, which may participate in membrane localization; a cysteine-rich domain, which may form zinc fingers; and two Src homology 3 (SH3) domains and a SH2 domain at its C-terminus, which are presumably involved in protein–protein interactions (Koch et al., 1991). Although structurally related to other guanine nucleotide exchange factor domains, the target of the Dbl domain has yet to be established (Bustelo et al., 1994; Gulbins et al., 1993; Khosravi-Far et al., 1994). Vav deficiency shows a role for the protein in efficient development of T and B cells (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995). In this context, Vav is constitutively phosphorylated in thymocytes (Gouy et al., 1995), while TCRζ/CD3 or TCRζ/CD3-CD4 ligation induces its phosphorylation in peripheral T cells (Bustelo and Barbacid, 1992; Margolis et al., 1992). T cell activation is defective in the absence of Vav expression (Tarakhovsky et al., 1995; Zhang et al., 1995). Further, the overexpression of the protein has been reported to augment TCRζ/CD3-induced interleukin-2 (IL-2) transcription (Motto et al., 1996; Wu et al., 1996). The SH2 domain of Vav can bind to a YESP motif within ZAP-70 (Katzav et al., 1994). A number of proteins have been reported to interact with Vav, including Grb-2, Shc, ZAP-70, ITK, and SLP-76 (Katzav et al., 1994; Onodera et al., 1996; Wu et al., 1996; Ye and Baltimore, 1994).

Recently cloned SLP-76 has been reported as a potential downstream link to Vav (Wu et al., 1996). The protein contains potential tyrosine phosphorylation sites in its N-terminus, a C-terminal SH2 domain, and a central proline-rich region that interacts with SH3 domain-containing proteins such as Grb-2. As in the case of Vav, SLP-76 is specifically expressed in hematopoietic cells and undergoes tyrosine phosphorylation upon TCR cross-linking (Bubeck Wardenburg et al., 1996; Motto et al., 1996; Onodera et al., 1996; Wu et al., 1996). Concordantly, receptor ligation induces the binding of Vav to SLP-76 via its SH2 domain (Onodera et al., 1996; Wu et al., 1996). Further, overexpression of SLP-76 augments TCR/CD3-induced IL-2 promoter activity and may synergize with Vav in augmenting IL-2 transcription (Wu et al., 1996). These SLP-76-mediated effects depend on the integrity of its SH2 domain (Motto et al., 1996), a domain that binds to two substrates at 62 kDa and 120 kDa (Motto et al., 1996). Taken together, these observations support an important role for the Vav–SLP-76 complex in IL-2 gene activation.

A key question concerns the identity of the tyrosine kinase and the sites that regulate Vav–SLP-76 binding and whether the interaction itself, or another property of these proteins, is required for TCR-mediated IL-2 production. In this report, we show that despite the presence of multiple kinases associated with TCR/CD3-CD4, only the ZAP-70 tyrosine kinase regulates the

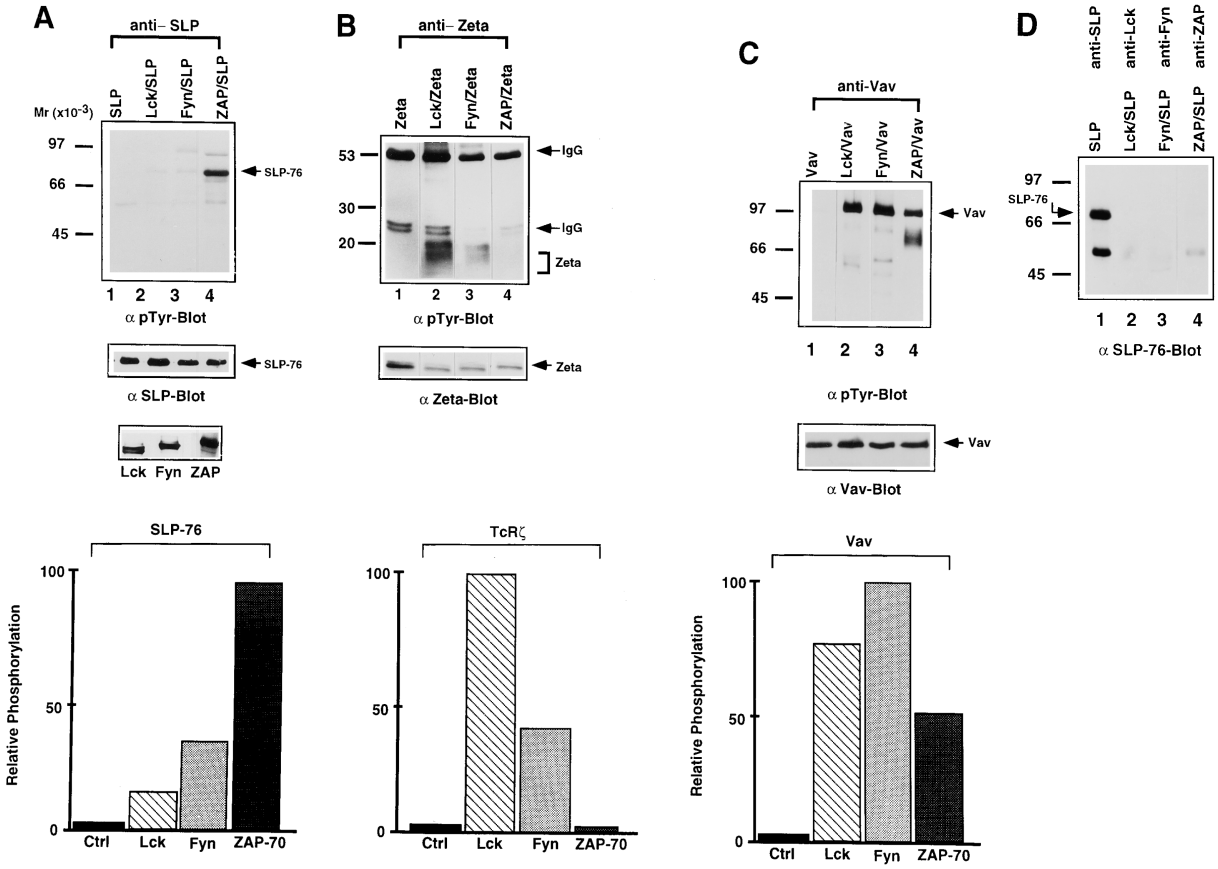

Figure 1. Phosphorylation of SLP-76 by the Protein-Tyrosine Kinase ZAP-70

(A) Differential phosphorylation of SLP-76 by the p56^{lck}, p59^{fyn}, and ZAP-70 kinases. SLP-76 was either expressed alone (lane 1) or in the presence of Lck (lane 2), Fyn (lane 3), or ZAP-70 (lane 4) in Sf21 cells. Lysates were prepared in 1% Triton X-100 lysis buffer, immunoprecipitated with anti-SLP-76 antibody (lanes 1–4), and immunoblotted with an anti-pTyr antibody.

(B) Differential phosphorylation of TCRζ by the p56^{lck}, p59^{fyn}, and ZAP-70 kinases. ζ was either expressed alone (lane 1) or in the presence of Lck (lane 2), Fyn (lane 3), or ZAP-70 (lane 4). Lysates were immunoprecipitated with anti-ζ antibody (lanes 1–4) and immunoblotted with an anti-pTyr antibody. Lanes show cells expressing ζ (lane 1), Lck/ζ (lane 2), Fyn/ζ (lane 3), and ZAP-70/ζ (lane 4).

(C) Phosphorylation of Vav by the p56^{lck}, p59^{fyn}, and ZAP-70 kinases. Vav was either expressed alone (lane 1) or in the presence of Lck (lane 2), Fyn (lane 3), or ZAP-70 (lane 4). Lysates were immunoprecipitated with anti-Vav antibody (lanes 1–4) and immunoblotted with an anti-pTyr antibody. Lanes show cells expressing Vav (lane 1), Lck/Vav (lane 2), Fyn/Vav (lane 3), and ZAP-70/Vav (lane 4). Equivalent levels of SLP-76, ζ, or Vav were present in all lanes as demonstrated by immunoblotting with an anti-SLP-76 MAb, anti-ζ MAb, or anti-Vav MAb, respectively. Densitometric analysis was carried out using the Scantjet laser scanner (Hewlett-Packard) of anti-pTyr binding to SLP-76 (A), ζ (B), or Vav (C)

(D) The absence of binding between SLP-76 and various kinases. Sf21 cells infected with virus carrying cDNA encoding SLP-76, Lck, Fyn, ZAP-70, or combinations of them were lysed, immunoprecipitated with anti-SLP-76 (lane 1), anti-Lck (lane 2), anti-Fyn (lane 3), and anti-ZAP-70 (lane 4) and immunoblotted with an anti-SLP-76 antibody. Positions of molecular mass markers (in kilodaltons) are indicated.

phosphorylation and interaction of SLP-76 with the Vav SH2 domain. p56^{lck} failed to phosphorylate SLP-76 significantly, while p59^{fyn} phosphorylated SLP-76 without inducing an interaction with Vav. Further, we show by various means that the two pYESP motifs of SLP-76 mediate binding to the Vav SH2 domain, with the first pYESP motif (residues 113–116) dominating. Finally, while Vav and SLP-76 augment IL-2, we demonstrate by using the T cell hybridoma DC27.10 that Vav–SLP-76 binding is not required for IL-2 production in all T cells.

Results

Recent studies have defined a downstream cascade from the TCRζ/CD3 complex involving ZAP-70 and Vav–SLP-76 (Katzav et al., 1994; Onodera et al., 1996; Wu et al., 1996). In transfection analysis, Vav and SLP-76 cooperate to increase IL-2 transcription (Wu et al., 1996) under circumstances in which the SH2 domain of Vav binds to SLP-76 (Onodera et al., 1996; Wu et al., 1996). A key event in the control of this interaction involves a kinase that phosphorylates the appropriate site. Although p56^{lck}, p59^{fyn}, and ZAP-70 associate with the CD4/CD8 or TCR/CD3 complexes, little is known regarding their specificities for downstream elements of the signaling pathway. To identify the kinase, we initially coexpressed SLP-76 with p56^{lck}, p59^{fyn}, and ZAP-70 in heterologous cells and assessed phosphorylation of the protein (Figure 1A). While ZAP-70 caused appreciable phosphorylation of SLP-76 (Figure 1A, lane 4), p56^{lck}

induced little SLP-76 phosphorylation (lane 2). $p59^{fyn}$ phosphorylated SLP-76 at some 20%–30% of that observed for ZAP-70 (lane 3). Specificity in this system was shown by the dramatic difference in the phosphorylation of TCRζ relative to SLP-76 by the different kinases (Figure 1B). Consistent with another report (Iwashima et al., 1994), TCRζ phosphorylation was mediated primarily by $p56^{lck}$ and to a lesser extent by $p59^{fyn}$ (Figure 1B, lanes 2 and 3, respectively). Further, no ZAP-70 phosphorylation of TCRζ was observed (lane 4). As a control for expression, SLP-76 and ζ expression were found to be equivalent in the presence of the different kinases and, further, each kinase was expressed at similar levels (Figure 1, middle panels). Kinase phosphorylation of SLP-76 occurred without any indication of physical binding between kinase and substrate (Figure 1D). Precipitation of $p56^{lck}$, $p59^{fyn}$, or ZAP-70 failed to coprecipitate SLP-76 (Figure 1D, lane 1 versus lanes 2–4).

For the purpose of comparison, we also examined the kinase responsible for the phosphorylation of Vav, a protein that becomes tyrosine phosphorylated in T cells as a consequence of TCR ligation (Bustelo and Barbacid, 1992; Margolis et al., 1992). In this case, all three kinases, $p56^{lck}$, $p59^{fyn}$, and ZAP-70, phosphorylated the substrate, although Fyn was consistently found to be the most efficient (Figure 1C, lane 3).

Given the apparent specificity of phosphorylation of SLP-76 by ZAP-70, it was of interest to assess whether ZAP-70 could regulate binding between Vav and SLP-76 (Figure 2A). Vav and SLP-76 were therefore coexpressed with the kinase and assessed for binding. No SLP-76 was present in anti-Vav precipitations in the presence of Lck (Figure 2A, lane 1) or in the absence of kinase (data not shown). However, in the presence of ZAP-70, anti-Vav precipitated significant levels of SLP-76, as detected by immunoblotting (Figure 2A, lane 3). As a control, anti-Vav blotting of lysates showed that equivalent amounts of Vav were expressed in the cells (Figure

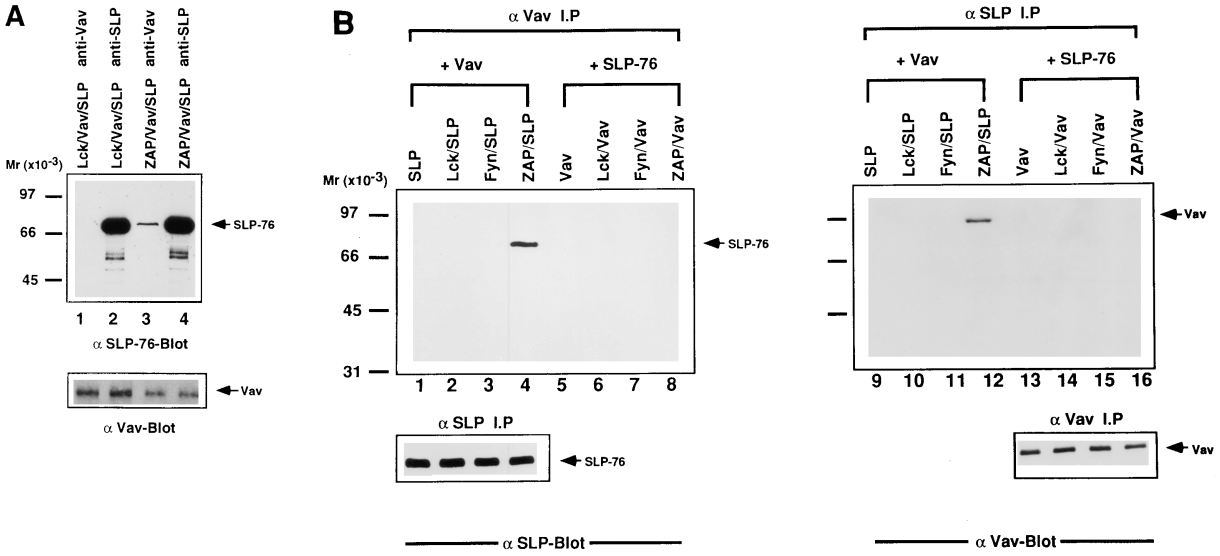

Figure 2. ZAP-70 Regulates Binding between Vav and SLP-76

(A) Coexpression of ZAP-70 induces binding between Vav and SLP-76. Sf21 cells were coinfected with Lck/Vav–SLP-76 (lanes 1 and 2) or ZAP-70/Vav–SLP-76 (lanes 3 and 4), lysed, immunoprecipitated with anti-Vav (lanes 1 and 3) or SLP-76 (lanes 2 and 4), and subjected to immunoblotting with anti-SLP-76 antiserum. Equivalent levels of Vav were expressed as demonstrated by immunoblotting of the cell lysate with an anti-Vav MAb.

(B) ZAP-70 phosphorylation of SLP-76, but not Vav, induces complex formation. Lysates from cells infected with virus carrying cDNAs encoding SLP-76 (lanes 1 and 9), Lck/SLP-76 (lanes 2 and 10), Fyn/SLP-76 (lanes 3 and 11), and ZAP-70/SLP-76 (lanes 4 and 12) were combined with the lysate of Sf21 cells expressing Vav. Lysates of cells expressing Vav (lanes 5 and 13), Lck/Vav (lanes 6 and 14), Fyn/Vav (lanes 7 and 15), and ZAP-70/Vav (lanes 8 and 16) were combined with the lysate of cells expressing SLP-76. After mixing lysates, immunoprecipitates using anti-Vav antibody (lanes 1–8) or anti-SLP-76 antibody (lanes 9–16) were prepared and subjected to immunoblotting with anti-SLP-76 antiserum (lanes 1–8) or anti-Vav antiserum (lanes 9–16). Cells expressing SLP-76, Lck/SLP-76, Fyn/SLP-76, and ZAP-70/SLP-76 were immunoprecipitated with anti-SLP-76 antibody and subjected to immunoblotting with anti-SLP-76 antiserum. Cells expressing Vav, Lck/Vav, Fyn/Vav, and ZAP-70/Vav were immunoprecipitated with anti-Vav antibody and subjected to immunoblotting with anti-Vav antiserum.

(C) Phosphatase digestion disrupts the Vav–SLP-76 complex. Sf21 cells coinfected with viruses carrying ZAP-70 and SLP-76 cDNAs (lanes 1–4) were lysed and treated with (lanes 2 and 4) or without (lanes 1 and 3) 50 U of alkaline phosphatase (Boehringer Mannheim) for 1 hr at 37°C. After dephosphorylation, the lysates were combined with lysates of cells expressing Vav, followed by immunoprecipitation with anti-Vav and immunoblotting with anti-pTyr antibody (lanes 1 and 2) or anti-SLP-76 antibody (lanes 3 and 4).

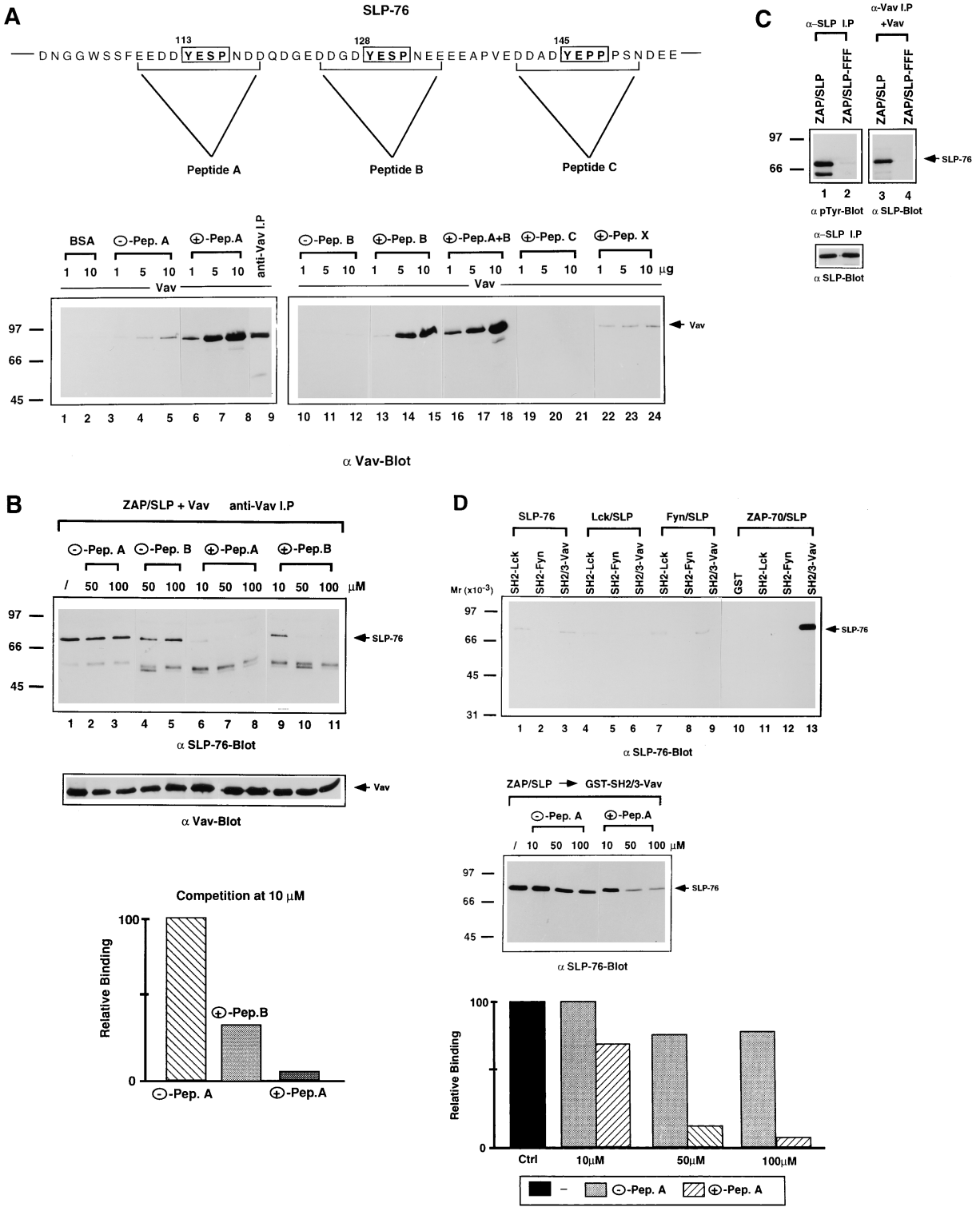

Figure 3. Vav SH2 Domain Binds to the YESP Motifs within SLP-76

(A) Peptides corresponding to the two pYESP motifs precipitate Vav. A depiction is shown of the SLP-76 antigen: residues 101–154 comprise peptide A (109–119) and peptide B (124–134), including the YESP motifs (113–116 and 128–131) and peptide C (142–151) with the YEPP motif (145–148) (upper panel). Lysates from Sf21 cells infected with virus carrying cDNA encoding Vav were incubated with different amounts of AminoLink Plus–coupled bovine serum albumin (BSA) (lanes 1 and 2), nonphosphorylated peptide A (lanes 3–5), phosphorylated peptide A (lanes 6–8), nonphosphorylated peptide B (lanes 10–12), phosphorylated peptide B (lanes 13–15), combined phosphorylated peptides A and B (lanes 16–18), phosphorylated peptide C (lanes 19–21), and an unrelated control phospho-peptide X with the sequence QKQFQPpYFIPIN (lanes 22–24). The precipitates were subjected to immunoblotting with anti-Vav antiserum (lanes 1–24). Lane 9 shows as a positive control immunoprecipitation with anti-Vav antibody (lower panel).

2A, lower panel). To verify that ZAP-70 phosphorylation of SLP-76 was required for the association, lysates from cells expressing SLP-76 and various kinases were mixed with lysates expressing Vav alone and assessed for binding. We previously established this system to demonstrate binding between other protein-binding partners (Raab et al., 1995; Raab and Rudd, 1996). Under these conditions, ZAP-70 phosphorylation of sites in SLP-76 was clearly able to induce binding to Vav (Figure 2B). Anti-Vav could precipitate SLP-76 (Figure 2B, lane 4), and anti-SLP-76 could precipitate Vav (lane 12). By contrast, neither Src kinase $p56^{lck}$ (which phosphorylated little SLP-76; Figure 2B, lanes 2 and 10) nor $p59^{fyn}$ (which phosphorylated the protein) could induce Vav–SLP-76 complex formation (lanes 3 and 11). Attempts to increase the sensitivity of detection by increasing amounts of lysate also failed to show complex formation using Src kinases (data not shown). This difference among kinases demonstrates the specificity of ZAP-70 in regulating the Vav–SLP-76 interaction. As a control, SLP-76 and Vav expression were monitored and found to be equivalent in the presence of different kinases as detected by immunoblotting (Figure 2B, lower panel). The same result was observed in multiple experiments.

Although the above data showed that ZAP-70 phosphorylation was sufficient to facilitate complex formation, we also tested whether the interaction occurred only in one direction. Despite the fact that Vav can also be phosphorylated by the various kinases (Figure 1C), its phosphorylation by Lck (Figure 2B, lanes 6 and 14), Fyn (lanes 7 and 15), and ZAP-70 (lanes 8 and 16) failed to allow for binding to SLP-76, a result consistent with previous reports (Onodera et al., 1996; Wu et al., 1996).

In an effort to verify that the binding was dependent on tyrosine phosphorylation, we assessed Vav–SLP-76 complexes for an ability to remain associated in the presence of alkaline phosphatase (Figure 2C). Under this regime, alkaline phosphatase both dephosphorylated SLP-76 (Figure 2C, lane 2 versus 1) and disrupted binding to Vav (lane 4 versus 3).

Although others have shown that the Vav SH2 domain recognizes phospho-SLP-76 (Onodera et al., 1996; Wu et al., 1996), the sites of recognition are not known. To define the region within SLP-76 that is phosphorylated by ZAP-70 and which mediates binding to Vav, we further investigated the Vav SH2-binding sites within SLP-76. Of particular interest are three possible sites within SLP-76, two YESP sites (residues 113–116 and 128–131), and one YEPP site (residues 145–148) that resemble the YESP site within ZAP-70 (Y315) (Katzav et al., 1994). To test which of these motifs bound to Vav, phosphorylated and nonphosphorylated peptides corresponding to each site EEDDYESPNDD (peptide A), DDGDYESPNEE (peptide B), and DDADYEPPPSN (peptide C) were initially coupled to AminoLink Plus beads and used to precipitate Vav from cell lysates (Figure 3A). Anti-Vav was then used in immunoblotting. Phosphorylated peptides A and B precipitated Vav in a manner correlated with the amount of peptide (Figure 3A, lanes 6–8 and 13–15), and peptide C failed to precipitate detectable protein (lanes 19–21). Increasing amounts of Vav were precipitated by 1 μg, 5 μg, and 10 μg of phosphorylated peptides A and B. Nonphosphorylated forms of the peptides A and B precipitated little material (Figure 3A, lanes 3–5 and 10–12). Additional negative controls included bovine serum albumin (Figure 3A, lanes 1 and 2) and an unrelated control phosphopeptide X (QKQFQPpYFIPIN; residues 212–223 of CTLA-4), which precipitate little antigen (lanes 22–24). Peptide A was routinely found to serve as a more efficient matrix for Vav binding. For example, at 1 μg, peptide A precipitated some 5- to 6-fold more Vav than did peptide B (Figure 3A). Combinations of peptide A and peptide B (0.5 μg plus 0.5 μg) also precipitated Vav (lanes 16–18), but generally at the same or slightly lower efficiency than peptide A alone. There is therefore no evidence for cooperativity when the two peptides are used together. These data show that the two SLP-76 pYESP motifs, but not the YEPP site, can bind to the Vav SH2 domain.

We next showed that phosphorylated peptides A and B could successfully dissociate Vav–SLP-76 complex that had been induced by ZAP-70 (Figure 3B). A similar approach has been used previously to identify sites of SH2 binding (Fantl et al., 1992; Roussel et al., 1991). Dissociation of SLP-76 from anti-Vav precipitates (Figure 3B, lanes 6–11) occurred in a concentration-dependent manner over a range of 10–100 μM (lanes 6–11). Nonphosphorylated peptides failed to compete for binding (lanes 1–5). Equivalent levels of Vav protein were present in all immunoprecipitates as detected by immunoblotting (Figure 3B, middle panel). In this regime, peptide A was again found to be more efficient (2- to 3-fold more effective) in competing than the peptide corresponding to the second pYESP motif (peptide B) (Figure

(B) Peptides corresponding to the two pYESP motifs compete for Vav–SLP-76 binding. Lysates from cells infected with viruses containing cDNAs encoding ZAP-70 and SLP-76 were combined with lysate from cells expressing Vav and incubated without (lane 1) or with different concentrations of nonphosphorylated peptide A (lanes 2 and 3), nonphosphorylated peptide B (lanes 4 and 5), phosphorylated peptide A (lanes 6–8), and phosphorylated peptide B (lanes 9–11). After immunoprecipitation with anti-Vav antibody, the precipitates were subjected to immunoblotting with anti-SLP-76 antiserum. Equivalent levels of Vav were present in all lanes as demonstrated by immunoblotting with an anti-Vav antibody (middle panel). Densitometric analysis of the competition experiment (lower panel).

(C) Mutation of pYESP/YEPP sites abrogates ZAP-70 phosphorylation and Vav binding. Sf21 cells were coinfected with ZAP-70 and SLP-76 (lanes 1 and 3) or ZAP-70 and SLP-76-FFF (lanes 2 and 4), lysed, immunoprecipitated with anti-SLP-76 (lanes 1 and 2) or combined with the lysate of Sf21 cells expressing Vav and immunoprecipitated with anti-Vav (lanes 3 and 4), and subjected to immunoblotting with anti-SLP-76 antiserum (lanes 1 and 2, lower panel; lanes 3 and 4, upper panel) or anti-Tyr (lanes 1 and 2, upper panel).

(D) (Top panel) Peptides corresponding to the first pYESP motifs can compete for Vav SH2 domain binding to SLP-76. Sf21 cells were infected with SLP-76 (lanes 1–3), coinfected with Lck/SLP-76 (lanes 4–6), Fyn/SLP-76 (lanes 7–9), or ZAP-70/SLP-76 (lanes 10–13), lysed in 1% Triton X-100, and subjected to precipitation with GST alone (lane 10), GST–Lck SH2 (lanes 1, 4, 7, and 11), GST–Fyn SH2 (lanes 2, 5, 8, and 12), or GST–Vav SH2/SH3 (lanes 3, 6, 9, and 13). The precipitates were separated on an SDS–polyacrylamide gel (10%) and subjected to anti-SLP-76 blotting. (Middle panel) ZAP-70/SLP-76-expressing Sf21 cells were lysed, incubated without (lane 1) or with different concentrations of nonphosphorylated peptide A (lanes 2–4) and phosphorylated peptide A (lanes 5–7), and subjected to precipitation with GST–Vav SH2/SH3 and immunoblotting with anti-SLP-76 antibody. (Lower panel) Densitometric profile of SLP-76 in GST–Vav SH3/SH3 precipitates.

3B, lower panel). Peptide concentrations as low as 10 μM reduced binding by greater than 90% (Figure 3B, lane 6; see histogram). At the same concentration, peptide B reduced binding by 40%–50% The concentration of peptides used to compete for binding is comparable with those used to block other SH2-mediated interactions, such as p85 SH2 binding to PDGF-R or CD28 (Fantl et al., 1992; Prasad et al., 1994).

Confirmation of Vav binding to the YESP motifs was observed in vivo using SLP-76 with mutations at the three sites (SLP-76-FFF) (Figure 3C). While coexpression of wild-type SLP-76 and ZAP-70 resulted in SLP-76 phosphorylation (lane 1), mutation of the three sites almost completely eliminated phosphorylation (lane 2). Equivalent amounts of wild-type and mutant SLP-76 protein were expressed (Figure 3C, lower panel). Elimination of these sites also prevented binding of Vav with SLP-76 in a mixed lysate experiment (Figure 3C, lane 4 versus 3). Given that the pYEPP site fails to bind to Vav (Figure 3A), these data demonstrate that ZAP-70 in vivo phosphorylation of one or both pYESP sites induces Vav–SLP-76 binding.

To confirm in our system that the SH2 domain of Vav mediates binding to SLP-76, we used glutathione S-transferase (GST) fusion proteins with the SH2/SH3 domains of Vav in precipitation assays. The Vav SH2/SH3 proteins specifically precipitated SLP-76 only when ZAP-70 was coexpressed with SLP-76 (Figure 3D, lane 13). Specificity was shown by the fact that other GST constructs such as GST (Figure 3D, lane 10), GST–Lck SH2 (lane 11), or GST–Fyn SH2 (lane 12) failed to precipitate the antigen. Since we used a construct that carried both the SH2 and SH3 domains, it was important to confirm that the phosphotyrosine motif was responsible for binding to the SH2 domain. We therefore examined the role of the SLP-76 pYESP motifs in Vav SH2 binding to ZAP-70-phosphorylated SLP-76. Under these conditions, we used phosphorylated peptide A and found it (50–100 μM) to compete effectively for SH2 Vav binding to SLP-76 (Figure 3D, lower panel). These data indicate that the ZAP-70 kinase phosphorylated the pYESP site that is needed for Vav SH2 recognition.

The cooperativity of Vav–SLP-76 in augmenting IL-2 transcription suggested that the interaction might be needed in IL-2 gene activation (Wu et al., 1996). To test this possibility, we investigated the interaction in a murine T cell hybridoma (DC27.10) that produces high levels of IL-2 in response to antigen receptor ligation (Yun-Cai et al., 1995; Zamoyska et al., 1989). The T cells expressed high levels of CD3 and showed a normal array of TCRζ and CD3 subunits as assessed by two-dimensional isoelectric focusing (data not shown). Significantly, although these cells produced high amounts of IL-2, with maximal levels of IL-2 observed at 10^{-9} M (Figure 4C), no binding between SLP-76 and Vav was detected as a consequence of TCR/CD3 ligation. Activation over varying lengths of time showed an increase in phosphorylation of Vav (Figure 4A, lanes 7–9) and SLP-76 (lanes 10–12) as detected by anti-phosphotyrosine (anti-pTyr) blotting. Under these conditions, no SLP-76 could be found in anti-Vav precipitations (Figure 4A, lanes 7–9) as detected by anti-pTyr blotting (upper panel) or immunoblotting using SLP-76 antiserum (middle panel). Conversely, no Vav was present in anti-SLP-76 precipitations (lanes 10–12). At least six experiments were conducted, each of which showed the same results. Earlier timepoints were also examined from as early as 20 s to 5 min (Figure 4B). Pervanadate activation also failed to induce an association as detected in anti-Vav (Figure 4D, lane 6) or anti-SLP-76 precipitates (lane 9). Both anti-pTyr and anti-SLP-76 were used in an attempt to detect the interaction (Figure 4D, upper and lower panels, respectively). As an important control, and as described by others (Onodera et al., 1996; Wu et al., 1996), the same experiments carried out using Jurkat cells showed the coprecipitation of SLP-76 with anti-Vav as detected by anti-SLP-76 blotting (Figure 4E, lanes 3 and 4). These observations make the important point that the Vav SH2 domain binding to SLP-76 per se is not required for TCR/CD3-induced IL-2 production in all T cells and suggests that the basis for Vav–SLP-76 cooperativity may be due to another biochemical feature of the SLP-76 and Vav signaling proteins.

Discussion

Previous studies have shown that both Vav and SLP-76 undergo tyrosine phosphorylation in response to TCR ligation, interact with each other, and augment NF-AT activity within the IL-2 promoter (Motto et al., 1996; Onodera et al., 1996; Wu et al., 1996). Each of these proteins may therefore participate in a signaling cascade initiated by ligation of the TCRζ/CD3 and CD4–$p56^{lck}$ complexes. A key question has concerned the identity of the kinase that regulates the interaction, and whether the interaction per se is required for TCRζ/CD3 induction of IL-2 production. Although $p56^{lck}$, $p59^{fyn}$, and ZAP-70 associate with CD4/CD8 and TCRζ/CD3 (Rudd et al., 1994; Samelson and Klausner, 1992; Weiss and Littman, 1994), remarkably little is known regarding their general substrate specificities and regulation of downstream signaling. $p56^{lck}$ and $p59^{fyn}$ can phosphorylate surface receptors such as TCRζ, CD5, and CD28, each of which possesses different tyrosine-based motifs (Iwashima et al., 1994; Burgess et al., 1992; Raab et al., 1994, 1995). In terms of downstream targets, the ability of Vav and SLP-76 to augment IL-2 and undergo complex formation provides an excellent model for examining the downstream target specificities of TCR- and CD4/CD8-associated kinases. Here, we show that of the three kinases, only ZAP-70 was capable of phosphorylating SLP-76 in a manner that induced Vav–SLP-76 binding (Figure 2). Specificity in our expression system was shown by the mirror image pattern produced by $p56^{lck}$ and ZAP-70 in the phosphorylation of SLP-76 relative to TCRζ (Figure 1). While SLP-76 was readily phosphorylated by ZAP-70, little phosphorylation was observed by Lck. Conversely, TCRζ was phosphorylated by $p56^{lck}$, with no observable phosphorylation by ZAP-70 (Figure 1), a result that is consistent with a previous report (Iwashima et al., 1994). Similar results were obtained with different levels of kinase expression (data not shown). By contrast, $p59^{fyn}$ phosphorylated SLP-76 at intermediate levels but, significantly, this phosphorylation failed to induce Vav–SLP-76 complex formation (Figures 1 and 2). This suggests that the kinase phosphorylates sites distinct from the pYESP sites and points to the distinct nature of this Src kinase relative to $p56^{lck}$.

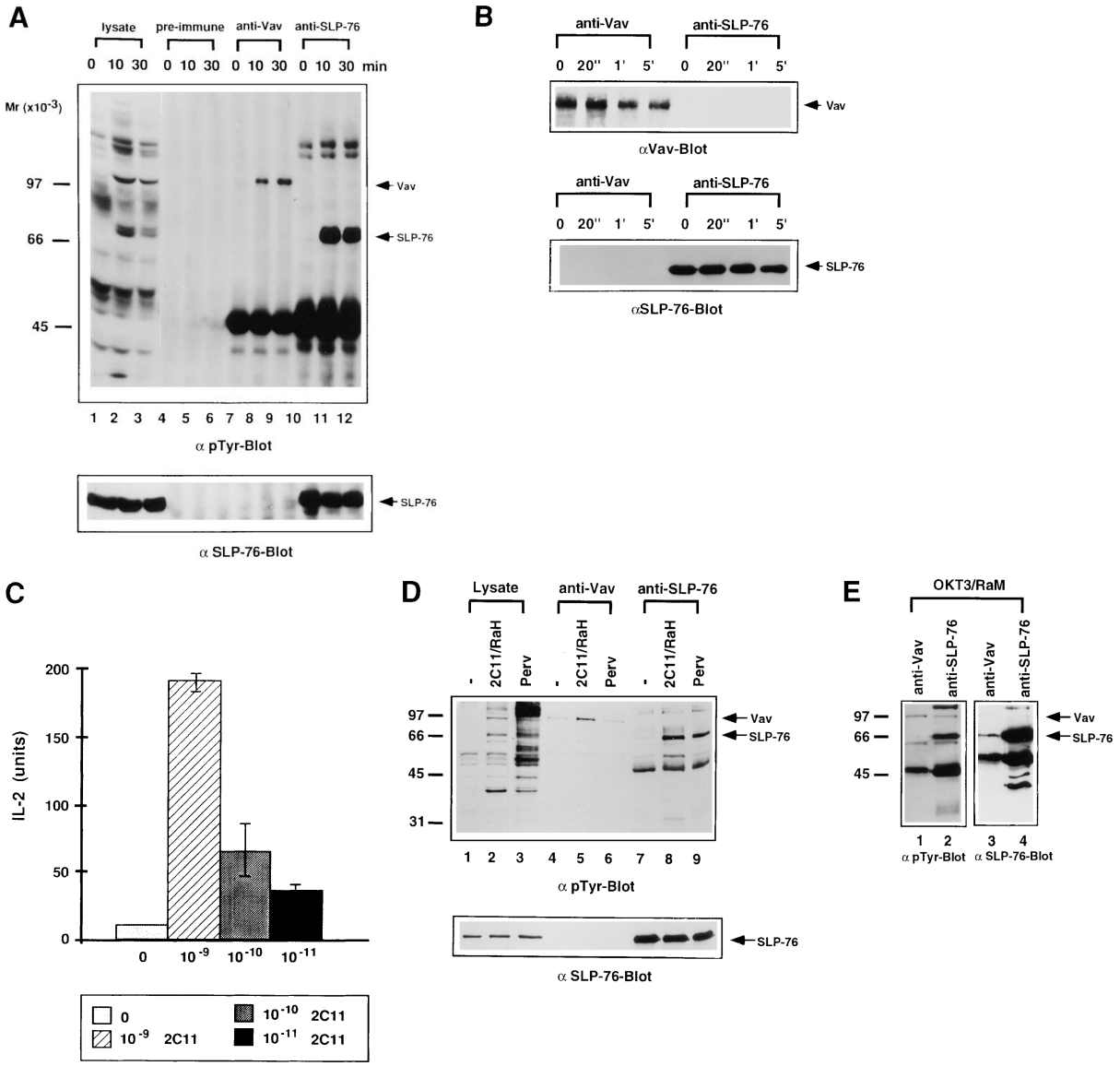

Figure 4. Production of High Levels of IL-2 in the Absence of Detectable Vav–SLP-76 Complex Formation

(A) No binding between SLP-76 and Vav after TCR/CD3 ligation. Precipitations from lysates of DC27.10 exposed to anti-CD3 (2C11) and rabbit anti-hamster (RaH) antibodies were subjected to immunoblotting with anti-pTyr antibody. Cells were cross-linked with anti-CD3 for 0 min (lanes 1, 4, 7, and 10), 10 min (lanes 2, 5, 8, and 11), and 30 min (lanes 3, 6, 9, and 12). Cell lysates (lanes 1–3) or precipitations with preimmune sera (lanes 4–6), anti-Vav antibody (lanes 7–9), or anti-SLP-76 antibody (lanes 10–12) are shown. Expression and precipitation of SLP-76 are demonstrated by immunoblotting with an anti-SLP-76 antibody.

(B) Anti-Vav and anti-SLP-76 precipitations from lysates of DC27.10 exposed to anti-CD3 antibody (2C11) and rabbit anti-hamster (RaH) antibody were subjected to immunoblotting with anti-Vav or anti SLP-76, respectively. Cells were cross-linked with anti-CD3 for 0 min (lanes 1 and 5), 20 s (lanes 2 and 6), 1 min (lanes 3 and 7), and 5 min (lanes 4 and 8).

(C) DC27.10 cells produce high levels of IL-2 after activation. IL-2 production in DC27.10 cells was analyzed by incubation with 2C11 at a concentration of 10^{-11} M to 10^{-9} M for 24 hr. Supernatants were harvested and analyzed for IL-2 in triplicates.

(D) No binding between SLP-76 and Vav after pervanadate activation. Precipitations from lysates of DC27.10 exposed to anti-CD3 (2C11) and rabbit anti-hamster (RaH) antibodies or pervanadate for 5 min at 37°C were subjected to immunoblotting with anti-pTyr antibody. Shown are unstimulated cells (lanes 1, 4, and 7) and cells stimulated with 2C11/RaH (lanes 2, 5, and 8) or pervanadate (lanes 3, 6, and 9). Cell lysates (lanes 1–3) or precipitations with anti-Vav antibody (lanes 4–6) or anti-SLP-76 antibody (lanes 7–9) are shown. Expression and precipitation of SLP-76 are demonstrated by immunoblotting with an anti-SLP-76 antibody.

(E) Binding between SLP-76 and Vav in Jurkat cells. Precipitations from lysates of Jurkat cells exposed to anti-CD3 antibody (OKT3) and rabbit anti-mouse (RaM) antibody for 5 min at 37°C were subjected to immunoblotting with anti-pTyr antibody (lanes 1 and 2) and anti-SLP-76 antibody (lanes 3 and 4). Precipitations with anti-Vav antibody (lanes 1 and 3) and anti-SLP-76 antibody (lanes 2 and 4) are shown.

Taken together, these data are consistent with a scenario in which $p56^{lck}$ regulates proximal events such as ZAP-70 recruitment, while ZAP-70 acts further downstream on targets such as SLP-76.

Previous work has shown that the SH2 domain of Vav binds to SLP-76; however, neither the kinase responsible nor the sites of binding were known. Here, we show that phosphorylated versions of peptides corresponding to both pYESP sites (residues 113–116 and 128–131) could successfully precipitate Vav and could compete

for the binding of Vav induced by ZAP-70 phosphorylation of SLP-76. A similar Vav SH2-binding motif (residues 315–318) with the pYESP sequence also resides within ZAP-70 (Katzav et al., 1994). By contrast, a third putative binding motif pYEPP (residues 145–148) failed to bind to Vav. Vav therefore exhibits specific binding to the pYESP motif, where a substitution of the serine residue with proline in the +3 position disrupts binding. Coexpression studies using the SLP-76 triple mutant confirmed that YESP sites are the principle in vivo sites for ZAP-70 phosphorylation (Figure 3C). Since the pYEPP site cannot bind to Vav (Figure 3A), one or both pYESP sites mediates in vivo binding to Vav (Figure 3C). The pYESP motifs are therefore possibly conserved in T cells as an general docking site for Vav. It is also noteworthy that the two pYESP sites of SLP-76 are not equal in mediating binding. The first pYESP motif was three to five times more efficient than the second motif in either precipitating Vav or competing for Vav–SLP-76 binding, suggesting that this motif will dominate in binding. Consistent with our findings, a recent report found that ZAP-70 could phosphorylate the YESP peptides in in vitro assays (Bubeck Wardenburg et al., 1996). Coexpression of truncated catalytic forms of $p56^{lck}$ with ZAP-70 was needed for significant SLP-76 phosphorylation, although an effect of phosphorylation on SLP-76 and Vav binding was not examined. In our system, ZAP-70 kinase alone sufficed to phosphorylate the sites needed for Vav SH2 binding to SLP-76, thus distinguishing its action from a potential back-regulatory effect of ZAP-70 on Lck. Taken together, these data demonstrate that ZAP-70 phosphorylates one or both pYESP motifs within SLP-76, which bind to the Vav SH2 domain. The consequences of SH2 binding are not known, although it could regulate Dbl activity in manner analogous to the effect of SH2 domain binding on SH-PTP2 phosphatase activity (Pluskey et al., 1995).

Previous studies have shown that, although they are structurally unrelated, in transfection analysis Vav and SLP-76 cooperate in augmenting TCR-mediated IL-2 transcription (Motto et al., 1996; Wu et al., 1996). This has lead to the interpretation that the physical interaction of Vav and SLP-76 regulates IL-2 production in a cascade from the TCRζ/CD3 complex. One complication in this model is the observation that the Vav SH2 domain binds to pYESP sites in both ZAP-70 and SLP-76. Hence, in this scheme, a cascade could involve sequential binding and dissociation of Vav from ZAP-70 to SLP-76. Alternatively, ZAP-70 and SLP-76 could compete with each other for Vav binding. To test the requirement for Vav–SLP-76 binding, we identified a T cell hybridoma (DC27.10) that produces high levels of IL-2 in the absence of detectable Vav–SLP-76 complex formation (Figure 4). No binding was observed despite anti-CD3 induction of Vav and SLP-76 phosphorylation. Examination of both early and later times following receptor cross-linking failed to show binding. Additional attempts to facilitate binding by means of pervanadate activation also failed to show binding (Figure 4D). At the same time, we could consistently show Vav–SLP-76 binding in Jurkat cells (Figure 4E). Our system offers the advantage that the role of the Vav–SLP-76 interaction could be examined in a T cell hybridoma without the need for transfection and overexpression of protein beyond endogenous levels. From this analysis one must conclude that Vav SH2 domain binding to SLP-76 is not strictly required for the ability of the TCRζ/CD3 complex to induce IL-2 production in all T cells.

Our findings may also provide an explanation for the apparent discordance between the levels of Vav–SLP-76 binding and IL-2 production in Jurkat cells expressing different isoforms of CD45 (McKenney et al., 1995; Onodera et al., 1996). It is important to note that although Vav–SLP-76 binding is not required for TCR-mediated IL-2 production, it could still amplify the response in T cells. Cooperativity between Vav and SLP-76 could also be related to their independent actions in downstream signaling. For example, independent signals may converge downstream in the regulation of a common target. In this context, the SLP-76 SH2 domain binds independently to two substrates at 62 kDa and 120 kDa (Motto et al., 1996). We are presently examining the downstream signaling pathways of Vav and SLP-76 in TCR/CD3-mediated T cell activation.

Experimental Procedures

Cells and Antibodies

The Spodoptera frugiperda (Sf) cell line IPLB-SF21 was obtained from Invitrogen and was propagated as a monolayer culture in Sf 900 insect medium (GIBCO BRL) supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin according to the procedure of Brown and Faulkner (1977). Viral infections were performed at a multiplicity of infection of 5 for protein production or of 0.1 for virus production. The murine T cell hybridoma, DC27.10 (gift of Dr. R. Zamoyska, Medical Research Council, London), or Jurkat cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum and 1% (w/v) penicillin–streptomycin at 37°C in an atmosphere containing 5% CO_2. Monoclonal antibodies (MAbs) to human Vav were provided by Dr. J. D. Griffin (Dana-Farber Cancer Institute, Boston, MA). Rabbit antisera against $p59^{fyn(T)}$ and $p56^{lck}$ were generated against synthetic peptides corresponding to residues 35–51 and 39–64, respectively. Antisera against ZAP-70 were provided by Dr. T. Mustelin (La Jolla Institute for Allergy and Immunology, La Jolla, CA). MAbs to SLP-76 were provided by Dr. P. R. Findell (Institute of Biochemistry and Cell Biology, Palo Alto, CA). Anti-pTyr MAb 4G10 was provided by Dr. T. Roberts (Dana-Farber Cancer Institute). Rabbit anti–mouse immunoglobulin was obtained from Dako Corporation (Carinteria, CA) and anti-murine CD3 (2C11) was from American Type Culture Collection.

Baculovirus Expression System

cDNAs encoding full-length $p59^{fyn(T)}$, $p56^{lck}$, ZAP-70, Vav, SLP-76, and the triple mutant of SLP-76 YYY/FFF (provided by Dr. R. Perlmutter, Seattle, WA; Dr. G. Raab, Boston, MA; Dr. B. Seed, Boston, MA; Dr. J. D. Griffin, Boston, MA; and Dr. P. R. Findell, Palo Alto, CA, respectively) were either amplified by PCR with specific oligonucleotides that included restriction sites for subcloning into the transfer vector pVL1392 or pVL1393 or directely cloned. S. frugiperda (Sf21) cells were then transfected with a mixture of linear wild-type baculoviral DNA (Invitrogen, San Diego, CA) and the pVL1392/pVL1393-DNA constructs and screened for recombinant virus plaques. Recombinant virus was purified from contaminating wild-type virus by two rounds of plaque purification.

Immunoprecipitation and Immunoblotting

Cells (1.5×10^6) were infected with the baculovirus encoding the different proteins. After 2 days, cells were harvested and lysed with 200 μl of lysis buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% [v/v] Triton X-100, 1 mM sodium vanadate, 1 mM PMSF, 1 mM leupeptin). Immunoprecipitation was carried out by incubation of the lysate with the antibody for 1 hr at 4°C, followed by incubation

with 50 μl of protein A–Sepharose beads (10% [w/v]) for 1 hr at 4°C. Immunoprecipitates were washed three times with ice-cold lysis buffer and subjected to SDS–PAGE. For immunoblotting, the immunoprecipitates from 1×10^6 to 2×10^6 cells were separated by SDS–PAGE and transferred onto nitrocellulose filters (Schleicher and Schuell, Keene, NH). Filters were blocked with 5% (w/v) skim-milk for 1 hr in Tris-buffered saline (pH 8.0) and then probed with the indicated antibody. Bound antibody was revealed with horseradish peroxidase–conjugated rabbit anti-mouse or donkey anti-rabbit antibodies using enhanced chemiluminescence (ECL; Amersham).

Peptide Competition and Binding Analysis

Peptides were synthesized and HPLC purified by the Molecular Biology Core Facility (Dana-Farber Cancer Institute). The sequences of the peptides used were as follows (with pY indicating the phosphorylated residue): phosphorylated peptide A, EEDDpYESPNDD; unphosphorylated peptide A, EEDDYESPNDD; phosphorylated peptide B, DDGDpYESPNEE; unphosphorylated peptide B, DDGDY ESPNEE; phosphorylated peptide c, DDADpYEPPPSN; phosphorylated peptide X, QKQFQPpYFIPIN (residues 212–223 of CTLA-4). For the competition analysis, peptides were added to mixed cell lysates and incubated for 2 hr at 4°C prior to immunoprecipitation and immunoblotting. For the binding analysis, peptides were coupled to AminoLink Plus gel beads (Pierce, Rockford, IL). The beads were added to the lysate and incubated for 2 hr at 4°C. The precipitates were then washed three times with ice-cold lysis buffer and subjected to SDS–PAGE and immunoblotting.

TCR/CD3-Mediated Activation of T Cells

To activate Jurkat and the DC27.10 murine T cell hybridoma, 50×10^6 cells were either treated with pervanadate for 5 min at 37°C or incubated with prewarmed RPMI media supplemented with 2% FCS, containing 5 μg/ml of 145-2C11 (anti-CD3ε) or anti-hCD3 (OKT3) and 10 μg/ml of the rabbit anti-mouse antibody at 37°C for varying lengths of time. Following activation, cells were then rapidly pelleted and solubilized in lysis buffer as described above.

IL-2 Assay

The supernatants derived from cell cultures were stored at −70°C and thawed before IL-2 assay. CTLL-2 cells were grown in the presence of recombinant human IL-2. They were washed three times and plated at 2500 cells per well in a 96-well plate. A series of 2-fold diluted supernatants were added to CTLL-2 cells. Cells were cultured for 24 hr followed by 18 hr of pulsing with 1 μCi of [^{3}H]thymidine before harvesting and counting. The amount of IL-2 in these supernatants was determined by comparison with the standard curve established with recombinant IL-2. The half-maximum of stimulation is defined as 100 U/ml, as described previously (Gillis et al., 1978).

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